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The second international standard for chlortetracycline *

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As supplies of the International Standard for Chlortetracycline were practically exhausted, it was replaced. The potency of the second international standard was defined on the basis of an international collaborative assay comprising 157 individual assays performed in 9 laboratories in 6 countries. The mean potencies obtained in the participating laboratories, although they varied by only 7%, were heterogeneous. However, the mean potency for all the laboratories combined did not differ significantly from that of the first international standard. The International Unit for chlortetracycline was therefore defined as the activity contained in 0.001 mg of the second international standard, corresponding to a potency of 1 000 IU/mg.

The WHO Expert Committee on Biological Standardization (1967) was informed that supplies of the first International Standard for Chlortetracycline were practically exhausted. Since active use was still being made of this standard and demand for it was considered likely to persist, material suitable for use as the second international standard was obtained and assayed against the first international standard.

MATERIALS FOR THE STUDY

The proposed second International Standard for Chlortetracycline

A sample of chlortetracycline hydrochloride, Batch No. 501-721B-36-2, weighing about 170 g was generously donated by Lederle Laboratories Division, American Cyanamid Company [3] through the good offices of Mr W. P. Jones of Cyanamid of Great Britain Ltd. The material, in two containers, each containing approximately 85 g, was received at the National Institute for Medical Research, London, in April 1966 and stored at −20°C, protected from moisture. The following data were supplied by the manufacturer:

Spectrophotometric assay (average result for 2 laboratories)	99.3%
Fluorometric assay (average result for 2 laboratories)	100.5%
Microbial assay [4]	1 090 " μg "/mg
Tetracycline content	0
Colour value (E 1%, 1 cm, 460 nm)	0.154
Volatile matter	0.34%
Rabbit skin test	Class 1
Heavy metals	26 mg/kg
Specific rotation $[\alpha]_D^{25}$	−237.8° as is −238.6° anhydrous
Chloride	6.92% ionic

In August 1966 the material was distributed into approximately 2 300 neutral glass ampoules so that each contained approximately 75 mg. The ampoules were closed with vented polyethylene plugs and dried over phosphorus(V) oxide *in vacuo* to constant weight. After they had been filled with pure dry nitrogen the ampoules were sealed by fusion of the glass, tested for leaks, and stored in the dark at −20°C.

The residual moisture content of the material in the ampoules, measured as loss in weight on drying to constant weight over phosphorus(V) oxide at 56°C and at a pressure of < 0.02 mmHg, was found to be 0.42% w/w (mean of 3 ampoules). When exposed to an atmosphere of 40% relative humidity at 20°C, the material in the ampoules picked up <0.15% w/w in 60 minutes. The residual oxygen concentration in the

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[4] When μg is placed between quotation marks (" μg "), it refers to a certain amount of activity (potency) and not to its accepted usage as a unit of mass.

Table 1. Details of assays and methods used

Laboratory No.	Assay method	Test organism	pH of assay medium	Diluent buffer	Dose range IU/ml	Incubation temperature	Response metameter	Total No. of assays	Valid assays[a]
1	Plate diffusion punch holes	*Bacillus subtilis* var. L2, No. 21	5.6–5.7	citrate pH 3.0	0.5–2.0		zone diameter	13	13
2	plate diffusion metal cylinders nephelometric	(see Table 2)					zone diameter	52	50
							square root of nephelometric reading	14	14 (3)
3	plate diffusion	*B. cereus* NCIB 8849			0.125–0.5	37°C	zone diameter	14	14
4	plate diffusion punch holes	*B. subtilis* ATCC 6633	6.5	phosphate pH 6.5	0.1–0.4	36–37°C	zone diameter[b]	4	4
							difference of zone diameters[c]	4	4 (1)
5	turbidimetric	*Staphylococcus aureus* ATCC 6538-P	7.0	phosphate pH 4.5	0.048–0.075	37°C	optical density	8	8
6	turbidimetric	*S. aureus* ATCC 6538-P	7.0	phosphate pH 4.5	0.047–0.077	37°C	optical density	8	8
7	plate diffusion paper disk	*B. cereus* ATCC 11778	7.0	phosphate	0.1–0.4	30°C	square of	7	6 (1)
		B. pumilus NCTC 8241	6.6	pH 5.8	0.625–2.5	37°C	zone diameter	15	14
8	plate diffusion beads	*B. cereus* NCTC 10320	6.0	$\frac{M}{26}$ KH_2PO_4	2.5–0.312	35°C	square root of area	8	8
		B. pumilus NCTC 8241	6.6		2.5–6.25	39°C		8	7
9	plate diffusion punch holes	*B. cereus* var. *mycoides* ATCC 9634	8.0	phosphate pH 4.5	0.4–0.16	30°C	zone diameter	8	7 (1)
total								163	157 (6)

[a] The numbers of assays with nonparallelism of borderline significance ($0.01 < P < 0.05$) are shown in parentheses.
[b] Referred to in the text as method 1.
[c] Referred to in the text as method 2.

ampoules was found to be 0.065% v/v (mean of 6 determinations).

The first International Standard for Chlortetracycline

The proposed second international standard was assayed against the first international standard established in 1953 (Humphrey et al.).

THE COLLABORATIVE ASSAY

Five ampoules of the proposed second international standard, numbered 1 to 5, together with 4 ampoules of the first international standard, numbered 1 to 4, were made available to each of the laboratories participating in the collaborative assay. These laboratories, which represent 6 different countries, are listed in Annex 1. Throughout the present article, each of them is referred to by a code number, which is not related to the order in which the laboratories are listed.

The participants were asked to design assays in a way that made it possible to carry out tests of linearity and parallelism of the log-dose response lines and also to ensure that each assay should provide, from its own internal evidence, an estimate of potency of the proposed second standard in terms of the existing standard and fiducial limits to that estimate. The participants were also invited to compare the two materials by any physical or chemical method of analysis that was being used in their laboratory.

STATISTICAL ANALYSIS OF RESULTS

The 9 participating laboratories provided the results of a total of 163 assays of the proposed second international standard in terms of the first international standard. These data were analysed statistically by standard methods for parallel line assays, relating response (or transformed response) to the logarithm of the dose. Five assays were discarded as invalid because the departure from parallelism term, in the analysis of variance, was significant at the 1% level. Assays were not necessarily rejected on the grounds of significant deviations from linearity of the log-dose response lines, for reasons discussed previously by Humphrey et al. (1953). However, a single assay from Laboratory 7 was omitted because not only was there significant curvature ($P < 0.001$) but also the estimated potency was discrepant from other values obtained by the same laboratory. In 6 of the 157 assays considered to be valid, the parallelism term was of borderline significance ($0.01 < P < 0.05$); thus the proportion of assays showing some degree of nonparallelism of the log-dose response lines was approximately the same as might have been expected to occur by chance.

A breakdown of assays by laboratory, giving details of the assay methods, the response metameters used in the analysis, and the validity of the assays is given in Table 1. For most assays there was a satisfactory linear relationship between the response (as measured) and the log dose, but for Laboratory 7 the square of zone diameter was more satisfactory and for Laboratory 8 the square root of zone area was more linear with the log dose. Both these laboratories arranged dilutions of the preparations under comparison in a Latin square design on large plates. Laboratory 3 also used large plates, whereas Laboratories 1 and 9 tested 3 dilutions of each preparation on Petri dishes. Some of the plate diffusion assays from Laboratory 2 were of a (3+3) design on Petri dishes; in others, 4 dilutions of each preparation were arranged in Latin squares on large plates. In the nephelometric assays from Laboratory 2, 3–7 dilutions were used and analysis was done after rejection of a minimum amount of data from the ends of the log-dose response lines, in order to obtain parallelism. This was also improved by the use of a square root transformation, but 10 assays still showed significant curvature at the 1% level. These assays were not excluded, since the potencies did not materially differ from those obtained when the untransformed data were analysed or when the responses for a more limited range of doses were used. Further details of the assays from Laboratory 2 are given in Table 2.

Laboratory 4 used two different designs on small plates, described here as methods 1 and 2. In method 1, 3 replicates of a single dilution of the proposed standard were tested alongside 3 replicates of the corresponding dilution of the current standard in each dish. In method 2, 3 positions on each plate were occupied by the same fixed dose of the standard, while one or other of the doses of either preparation was tested on the other half of the plate. In the latter assays, the deviation of the mean zone diameter for the dose being tested from the mean zone diameter for the fixed dose was used as the response metameter.

For the basic analysis of the turbidimetric assays performed in Laboratories 5 and 6, optical density was related to the log dose.

Table 2. Details of assays and methods used by Laboratory 2

Assay method	Test organism	pH of assay medium	Diluent buffer	Dose range in IU/ml	Incubation temperature	Response metameter	Total No. of assays	Valid assays [a]	Potency ratio w/w	Confidence limits (P = 0.95)
plate diffusion	*B. cereus* ATCC 9634	6.5–6.6	phosphate pH 4.5	0.5–0.125	30°C	zone diameter	24	23	0.976	0.960–0.993
	B. pumilus NCTC 8241	6.5–6.6	phosphate pH 6.0	4.0–2.0	35°C		8	8	0.971	0.953–0.989
	B. subtilis ATCC 6633	6.6	phosphate pH 6.0	8.0–1.0	35°C		5	5	0.974	0.942–1.007
	Sarcina lutea ATCC 9341	6.5–6.6	phosphate pH 6.0	13.5–4.0	35°C		15	14	0.990	0.971–1.009
nephelometric	*Klebsiella pneumoniae* ATCC 10031	7.0	phosphate pH 6.0	0.656 –0.100	37°C	square root of nephelometric reading	7	7	0.972	0.940–1.006
	S. aureus ATCC 6538-P	7.0	phosphate pH 6.0	0.3375–0.020	37°C		7	7 (3)	0.968	0.908–1.033

[a] The numbers of assays with nonparallelism of borderline significance ($0.01 < P < 0.05$) are shown in parentheses.

Individual log potencies (M_i) were tested for homogeneity within each laboratory and each assay method, using the formula:

$$\chi^2 = \Sigma W_i (M_i - \bar{M})^2$$

where the weights (W_i) are the reciprocals of the variances of the log potencies and $\bar{M}$ is the weighted mean potency.

For Laboratories 1, 3, 6, 8, and 9, potency values were homogeneous—i.e., the probability of χ^2 was greater than 0.05 and the weighted means (expressed as potency ratios, on a weight-to-weight basis) are given in Table 3, together with their confidence limits, based on the total weights.

Where the χ^2 test indicated heterogeneity, unweighted geometric mean potencies were evaluated and confidence limits were estimated by the " direct " method—i.e., by calculating the variances direct from the distributions of individual log potencies.

For Laboratory 2, all 50 potencies obtained by plate diffusion methods were homogeneous, and the weighted means are given in Tables 2 and 3. For the nephelometric assays the potencies were heterogeneous for each test organism, but the two direct estimates were homogeneous with each other and with the results for the plate diffusion assays and so were combined to give an overall value for the laboratory.

For Laboratory 4, the assays done by method 1 were homogeneous. With method 2, however, there was heterogeneity within the set and with the potency for method 1. For this reason no overall estimate for the laboratory was derived, the values in Table 3 representing the weighted mean for method 1 and the unweighted mean for method 2. Laboratory 5 carried out 2 assays per day for 4 days. As there was homogeneity within days, but not between days, the overall mean was calculated direct from the 4 daily means. There was some heterogeneity among the *Bacillus pumilus* assays provided by Laboratory 7, but when assays were grouped according to the days on which they had been carried out the χ^2 between days was only of borderline significance and the weighted mean for the 4 days was homogeneous with the results obtained from the *Bacillus cereus* assays.

The log potencies for each laboratory, weighted either from the internal evidence of the assays or from the variation between assays (as described above) were found to be heterogeneous ($P < 0.001$). This did not appear to stem from the results of any particular laboratory or assay method, so the final value for the study of 1.004 with confidence limits

Table 3. Potencies obtained by different laboratories

Laboratory No.	No. of assays	Potency ratio w/w	Confidence limits (P = 0.95)
1	13	1.049	1.038–1.060
2	plate diffusion (50)	0.978	0.968–0.988
	nephelometric (14)	0.971 [a]	0.943–1.001
	64	0.977	0.968–0.987
3	14	0.999	0.986–1.011
4	method 1 (4)	0.984	0.972–0.996
	method 2 (4)	1.054	1.015–1.095
5	8	1.013 [b]	0.995–1.032
6	8	1.027	1.020–1.034
7	*B. cereus* (6)	1.025	1.012–1.038
	B. pumilus (14)	1.042 [c]	1.034–1.050
	20	1.037	1.030–1.044
8	*B. cereus* (8)	1.007	0.998–1.016
	B. pumilus (7)	0.992	0.979–1.005
	15	1.004	0.997–1.012
9	7	1.005	0.993–1.017

[a] Based on weighted means for 2 test organisms.
[b] Unweighted mean of 4 daily weighted means.
[c] Based on 4 daily weighted means.

(P = 0.95) of 0.998–1.011 was obtained as a direct estimate from the distribution of the 157 log potencies considered to be valid.

As the International Standard for Chlortetracycline had a potency of 1 000 IU/mg, the proposed second international standard was estimated to contain 1 004 (998–1 011) IU/mg.

DISCUSSION

Considering the putative purity of the two materials being compared in this collaborative assay, the variations in potency obtained by the different laboratories is surprising. A much greater range of assay conditions was used than is usual in a collaborative assay, but it is difficult to see any relationship between the variations in potency and the variations in assay conditions. Laboratory 2 made an extensive comparison of the two materials by a variety of methods and techniques encompassing most of those used by the other participating laboratories. The results showed good agreement between the different methods, again suggesting that the recorded variations in assay conditions did not influence the potency ratios obtained.

Table 4. Summary of results obtained by chemical and physical methods for the comparison of purity of the International Standard for Chlortetracycline (IS) and the Proposed Second International Standard (PSIS)

Laboratory No.	Ratio of purity [a]	Method used
3	1.00	Chiccarelli et al. (1957)
4	0.998 (0.994–1.001)	Extinction in N. H_2SO_4 λ = 274 nm
	0.999 (0.993–1.005)	Extinction in N. H_2SO_4 λ = 274 nm after heating
9	1.005 (0.998–1.012)	Grove & Randall (1955)

[a] Ratio PSIS : IS with 95 % confidence limits.

Although mean potency ratio values for the different laboratories were not statistically consistent, they varied by only about 7%, whereas potency ratios from the 157 individual assays extended over a range of approximately 30%. All the laboratories except one assayed solutions on the day they were prepared, but it is possible that there were differences in the time that elapsed between the solution of the samples and their use in the assay, which might account for some of the variations in potency.

No significant difference between the activity of the proposed second standard and that of the first standard was demonstrated, and it was suggested to the participating laboratories that the new standard should be assigned a potency of 1 000 IU/mg.

The participating laboratories were invited to compare the existing and proposed standards by any chemical and physical methods they were currently using. Such comparisons were made by 3 laboratories and their results are summarized in Table 4. The ratios obtained agreed closely between the laboratories and supported a conclusion of equal activity for the materials compared.

In accordance with the authorization of the WHO Expert Committee on Biological Standardization (1967), the proposed material was established as the Second International Standard for Chlortetracycline and, with the agreement of the participants in the study, the International Unit for chlortetracycline was defined as the activity contained in 0.001 mg of the Second International Standard for Chlortetracycline.

RÉSUMÉ

DEUXIÈME ÉTALON INTERNATIONAL DE CHLORTÉTRACYCLINE

Le Comité OMS d'experts de la Standardisation biologique (1967) ayant noté que les stocks de l'étalon international de chlortétracycline étaient en voie d'épuisement, avait demandé au National Institute for Medical Research, de Londres, d'établir un deuxième étalon international.

Du matériel approprié en vue du remplacement du premier étalon a été obtenu et soumis à un titrage comparatif dans neuf laboratoires de six pays. Au total, 157 épreuves ont été pratiquées, par diffusion et turbidimétrie, sur une série de micro-organismes. L'analyse statistique a fait ressortir l'hétérogénéité des estimations de l'activité dans quatre laboratoires, de même que des variations de l'activité moyenne de la préparation proposée suivant les laboratoires, mais dans une proportion ne dépassant pas 7%. La cause de ces variations intra- et inter-laboratoires n'a pas été déterminée. L'examen des résultats d'ensemble a montré que l'activité de la préparation proposée ne différait pas sensiblement de celle du premier étalon.

En conséquence, les participants ont décidé de constituer le matériel proposé en deuxième étalon international de chlortétracycline, dont l'activité a été fixée à 1000 UI par milligramme. L'unité internationale de chlortétracycline a été définie comme l'activité de 0,001 mg du deuxième étalon international de chlortétracycline.

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Annex 1

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